Isolation of glycoproteins from brown algae.

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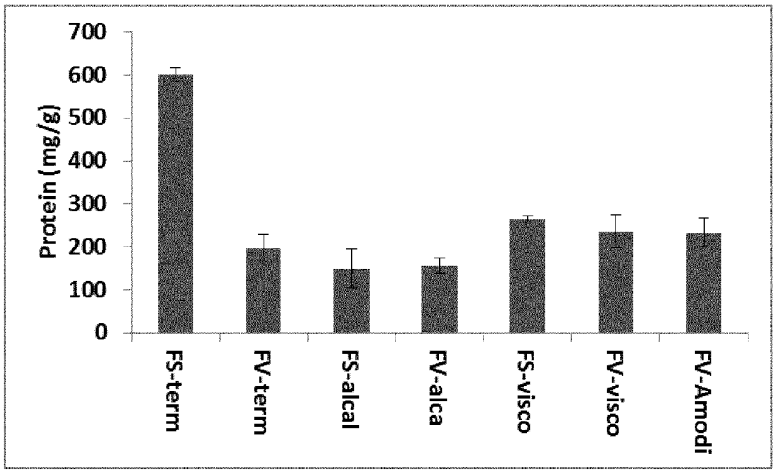

FIGURE 3

(57) **Abstract**: The present invention relates to a novel process for the isolation of unique anti-oxidative glycoproteins from the pH precipitated fractions of enzymatic extracts of brown algae. Two brown seaweeds viz, Fucus serratus and Fucus vesiculosus were hydrolysed by using 3 enzymes viz, Alcalase, Viscozyme and Termamyl and the glycoproteins were isolated from these enzyme extracts.

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TITLE: ISOLATION OF GLYCOPROTEINS FROM BROWN ALGAE

Field of the invention

The present invention relates to naturally occurring antioxidants, their isolation from seaweeds and use as for example antioxidants. More particular, the invention relates to a method for isolation of a mixture of anti-oxidative glycoproteins from brown algae. The isolated glycoproteins have very good Fe^{2+} chelating activity, which is better than EDTA, and other anti-oxidative activities comparable to commercial antioxidants.

Background of the invention

For centuries, seaweeds have been utilized traditionally as food supplements and for various medicinal purposes. Research in natural products of marine algae has made significant advances in recent years and marine algae have been shown to produce a variety of secondary metabolites of potential medicinal value. The secondary metabolites synthesized by seaweeds demonstrate a broad spectrum of bioactivity including antioxidant, anti-inflammatory, anticancer, anti-diabetic and anti HIV activity. A number of potent anti-oxidative compounds have been isolated and identified from different types of seaweeds, including phlorotannins, sulphated polysaccharides, carotenoid pigments such as fucoxanthin and astaxanthin, sterols, catechins and proteins.

The high degree of structural complexity and rigidity of the algal cell wall is a major obstacle to the efficient extraction of the intracellular bioactive constituents. Conventional water and solvent extractions have several drawbacks such as low selectivity, low extraction efficiency, solvent residue and environmental pollution. As an alternative technology, enzyme-assisted extraction has attracted considerable interest. Enzymatic extraction has also been reported to increase the extractability of bioactive compounds from several brown algae (Wijesinghe and Jeon, (2012). The cell wall degrading enzymes help to weaken or disrupt the cell wall structure, break down complex interior storage materials, thereby facilitating the release of intracellular bioactive compounds in an extract from algal biomass. The hydrolytic breakdown of high-molecular-weight (HMW) polysaccharides and proteins may contribute to enhanced anti-oxidative activities, which are mainly attributed to the content of polyphenols and carotenoids (Wijesinghe and Jeon, (2012)).

Glycoproteins are proteins that contain oligosaccharide chains (glycans) covalently attached to polypeptide side-chains. The carbohydrate is attached to the protein in a co-translational

or posttranslational modification by a process known as glycosylation. The carbohydrate can make up anywhere from less than one percent to more than 80 percent of the total glycoprotein mass. The presence of sugars makes glycoproteins far more hydrophilic and is often essential for the proper folding of the protein into its tertiary structure and function.

Glycoproteins isolated from macro-algae have already been implicated in the control of apoptosis and cell proliferation, as antioxidant, for DNA and liver protection (Kim et al., 2012, Hwang et al, 2008). Kim et al. (2012) isolated a single glycoprotein from *Saccharina japonica* and Hwang et al. (2008) a glycoprotein from *Hizikia fusiformis* by water extraction and ethanol precipitation. The method of isolation of glycoproteins from macro-algae described in literature involves ethanol precipitation of water extracts followed by ammonium sulphate precipitation of the supernatant. During the ethanol precipitation steps both carbohydrates and proteins get precipitated and some of the glycoproteins may also be lost in the precipitate.

Takeo Yoshiie et al. (2012) analysed the structural features of N-glycans linked to glycoproteins isolated by ion-exchange from pepsin digests of seaweeds.

Rice, E.L. and R.K. Crowden (1987) extracted enzymes of glycoprotein nature from brown algae by use of ion exchange followed by analysis of the carbohydrate and the enzyme parts.

The present invention differs from other reported methods of isolating intracellular bioactive compounds with anti-oxidative properties, as the glycoproteins are isolated from enzymatic extracts of brown algae and devoid of lipids, phenolic compounds and free polysaccharides. The present invention thus deals with the isolation of a unique kind of glycoproteins from pH precipitated fractions of enzymatic extracts of brown algae.
The isolated glycoproteins show very good anti-oxidative activity especially iron chelating activity.

Summary of the invention

The present invention relates to a novel process for the isolation of unique anti-oxidative glycoproteins, e.g. a mixture different glycoproteins, i.e. a glycoprotein composition from pH precipitated fractions of enzymatic extracts of brown algae.

In a first embodiment, the invention concerns a method for isolation of glycoproteins from brown algae, comprising treating an aqueous solution of brown algae powder with one or more hydrolytic enzymes followed by a precipitation of the glycoproteins at low pH, preferably a pH below 3.

More particular, the present method comprises the steps of removing lipids, phenolic compounds and free carbohydrates in order to obtain a glycoprotein composition substantially free of interfering compounds.

Thus, in a second embodiment, the method includes the following steps:

a) providing a powder of dried brown algae,
b) treating an aqueous solution of brown algae powder with a hydrolytic enzyme or combination of hydrolytic enzymes,
c) removing lipids, e.g. by extraction with hexane,
d) removing phenolic compounds, eg. by extraction with ethyl acetate,
e) adjusting pH of the aqueous solution to 1-3 to precipitate glycoproteins, and
d) removing free carbohydrates, e.g. by extraction with ethanol:diethyl ether, ethanol and anhydrous diethyl ether.

The hydrolytic enzymes are selected from one or more peptidases, such as endo-peptidases, one or more carbohydrases, such as alpha-amylases, or a combination thereof. Multi-enzyme complexes may also be applied.

To illustrate the present invention, seaweeds, viz, the brown algaes *Fucus serratus* and *Fucus vesiculosus* were isolated from Danish seawaters, rinsed, freeze-dried and powdered and the powder solubilized and hydrolysed using different enzymes and the glycoproteins isolated from these enzyme extracts by lowering the pH until precipitation of the glycoproteins.

During the process, lipids, phenolic compounds and free polysaccharides are removed, for example by extraction with organic solvents.

The isolated glycoproteins show very good antioxidative activity especially iron chelating activity. Different combinations of seaweed and enzyme may lead to different glycoprotein products which may possess different properties.

Brief disclosure of the drawings

Figure 1 shows a diagram over the isolation of glycoprotein from pH precipitated fractions of enzymatic extracts of brown algae.

Figure 2 shows a diagram over a modified method for the isolation of glycoprotein from pH precipitated fractions of enzymatic extracts of brown algae.

Figure 3 shows the protein content of the glycoprotein as obtained by the agarose method.

Figure 4 shows total soluble carbohydrate in obtained glycoproteins as determined by the phenol –sulphuric acid method.

Figure 5 shows reducing sugar content in the obtained glycoproteins as determined by the DNS method.

Figure 6 shows the sulfate content in the obtained glycoproteins as determined by the agarose method.

Figure 7 shows the % DPPH radical scavenging activity of the obtained glycoproteins.

Figure 8 shows the Fe^{2+} chelating activity of the obtained glycoproteins.

Figure 9 shows the reducing power (OD at 700nm) of the obtained glycoproteins.

Figure 10 shows the % inhibition of lipid oxidation of the obtained glycoproteins in the liposome model system.

Figure 11 shows the SDS page electrophoresis pattern of isolated glycoproteins subjected to periodic acid oxidation and subsequent Alcian Blue and silver staining. b) isolated glycoproteins subjected to silver staining alone showing protein component . Lane 1 and 9: marker; Lane 2: FS-term; Lane 3: FV-term; Lane 4: FS-Alcal; Lane 5: FV-Alcal; Lane 6: FS-visc; lane 7: FV-visc; Lane 8: Fv-Alcal modified method.

Detailed description of the invention

The present invention relates to a novel method for the isolation of unique anti-oxidative glycoproteins from the pH precipitated fractions of enzymatic extracts of brown algae. Brown seaweeds collected from Danish seawaters were used as the raw material for the glycoprotein isolation to illustrate the present method. These seaweeds were hydrolysed by different enzymes and subjected to removal of lipids, phenolic compounds before precipitation by adjusting pH to below 3. These pH precipitated fraction were subjected to glycoprotein isolation by removing non-protein matter and free polysaccharides.

In a first aspect, the present invention relates to a method for isolation of glycoproteins from brown algae, comprising the steps of a) providing a powder of brown algae, b) treating

an aqueous solution of the brown algae powder with a hydrolytic enzyme or a combination of hydrolytic enzymes, and c) precipitating glycoproteins from the enzymatic extracts of the brown algae powder at a low pH.

The pH in step c) is preferably between 1 and 3, and may be below 2, for example between 1.5 and 2. Lowering the pH may be achieved by any suitable acid, such as HCl.

Seaweed powder can be purchased as a commercial product or prepared from living seaweed prior to the enzymatic treatment. Immediately after collecting seaweeds from the ocean, pre-treatment is started by rinsing the seaweeds in, e.g. tap-water, and placing the rinsed seaweeds in a freezer until further use. When used, the seaweed samples are freeze-dried and ground to a fine powder and passed through a sieve to get a uniform powder. If not used immediately, the powdered seaweeds are preferably stored at -80 ^{o}C under vacuum packing.

As illustrative for the present invention, the seaweeds are the brown algae *Fucus vesiculosus* and *Fucus serratus*_samples_collected from the sandy beaches of Hou havn (55^{o}54'39N 10^{o}14'59E) in Denmark

In order to obtain the glycoproteins in a relatively pure form, the method may preferably comprise removal of lipids, phenolic compounds and free carbohydrates (mono-, di-, oligo- and polysaccharides). The removal is preferably an extraction of the lipids, e.g. by extraction with hexane or a similar solvent, of the phenolic compounds, e.g. by extraction with ethyl acetate or a similar solvent, and of free carbohydrates, e.g. by extraction with ethanol:diethyl ether, ethanol and/or anhydrous diethyl ether.

According to the present invention, the lipids and the phenolic compounds are removed after the enzymatic treatment, while the free carbohydrates are removed after precipitation of the glycoproteins.

Accordingly, the present method comprises the following steps:

a) providing a powder of dried brown algae,
b) treating an aqueous solution of brown algae powder with a hydrolytic enzyme or combination of hydrolytic enzymes,
c) removing lipids, e.g. by extraction with hexane,
d) removing phenolic compounds, eg. by extraction with ethyl acetate,
e) adjusting pH of the aqueous solution to 1-3 to precipitate glycoproteins, and

d) removing free carbohydrates, e.g. by extraction with ethanol:diethyl ether, ethanol and anhydrous diethyl ether.

In a modified method which was developed for industrial production of glycoproteins according to the present invention, some of the freeze drying and centrifugation steps may be removed compared to the full method, as illustrated in the examples. The removal of some of the freeze drying and centrifugation steps will change the composition of the final glycoprotein products, but lead to a more simple and time and cost effective method.

The hydrolytic enzymes to be used in the present method are selected from one or more peptidases, one or more carbohydrases. A combination of different peptidases and carbohydrases may also be applied. The enzymatic hydrolysis of the powder is performed in a suitable buffer at the pH optimum for the used enzyme or mixture of enzymes. If necessary, the enzymatic reaction may be performed in more steps, for example if pH optimum is different for the different enzymes to be used.

As peptidases, both exo- and endo-peptidases may be used, alone or together. Preferable, an endo-peptidase, such as for example the commercial Alcalase, is used. Similar peptidases are known in the art and may be used in a similar way.

Many carbohydrases exist which may be used in the present method. However, the enzyme may preferably be an amylase and more preferably an alpha-amylase, for example a commercial alpha-amylase, such as Termamyl, or similar amylase preparation known in the art.

Alternative to peptidases and carbohydrases, as the enzyme, a multi-enzyme complex, such as Viscozyme may be applied. Many similar complexes are known in the art or may be composed for this purpose and may be applied in a similar way.

The choice of enzyme or combination of different enzymes may affect to extraction efficiency and thus also the final composition of glycoproteins in the glycoprotein composition after removal of other compounds.

The glycoproteins are precipitated and all other components present in the enzymatic extract removed in the following way. The enzymatic extracts are homogenized in an aqueous media, e.g. water, in order to get a uniform solution. Lipids present in the aqueous solution are extracted with an organic solvent such e.g. n-hexane and the hexane phase

discarded. The aqueous phase is extracted in a suitable solvent, e.g. ethyl acetate, in order to remove phenolic compounds. The pH of the aqueous phase is adjusted to about 5 and optionally followed by further extractions in the same way to remove phenolic compounds. The pH of the aqueous phase is then adjusted to a value between about 1.5 and about 2 to precipitate protein components. The remaining aqueous phase may be extracted again as described above in order to remove any residual phenolic compounds, followed by removal of any leftover of the extraction solvent from the water phase, for example by evaporation in a rotary evaporator. The precipitate is collected from the water phase, e.g. by centrifugation and freeze dried. Cold urea is added to the pH precipitate and pH adjusted to about 8.5. In order to remove any non-proteinacious matter present at this stage, the solution is stirred in a magnetic stirrer at low temperature for a while before centrifugation and discarding of any pellet. A TCA solution is added to make a final concentration of 5-10% V/V TCA which precipitates glycoprotein. The supernatant is discarded and the precipitate dissolved in water. The pH is adjusted (about 7.2 to about 7.5) such that the precipitate dissolves in water followed by another centrifugation to remove any un-dissolved matter. The supernatant is filled in a dialysis membrane and dialyzed against water for several hours (molecular cut-off dialyzing tubes about 1kDa) to remove any small molecules and salts. The pH of the dialyzed sample is adjusted to about 1.5-2.5 and centrifuged, followed by washing of the precipitate with a suitable solvent (e.g. ethanol:diethyl ether (3:1)) to remove unbound carbohydrates. The washing step may be repeated. Optionally the pellet is washed with absolute ethanol and anhydrous diethyl ether before being dried.

In a second aspect, the present invention relates to a glycoprotein composition from brown algae obtainable in a method according to the first aspect of the present invention. Such glycoprotein compositions and fractions thereof may be used as anti-oxidative agents in consumer's products such as for example foods and cosmetic and in pharmaceuticals.

The glycoproteins isolated according to the present method were subjected to evaluation for anti-oxidative activity. The anti-oxidative activity of the glycoproteins were determined by four different methods viz, Fe2+ chelating activity, reducing power, DPPH radical scavenging activity and inhibition of lipid oxidation in a liposome model system. The different methods are explained in the examples. The isolated glycoproteins showed >90% iron chelating activity even when tested in as low concentration as 0.01 mg/mL. At this concentration EDTA, one of the most powerful synthetic metal chelators only had approximately 20 % iron chelating activity. Lipid oxidation in foods and biological systems are often catalysed by free iron or heme-iron. Hence, the high metal chelating activity of the glycoproteins suggests that they may be able to efficiently inhibit lipid oxidation in

different food and biological systems. DPPH radical scavenging activity at 5 mg/ml was comparable to that of the synthetic antioxidant BHT commonly used in the food industry.

The reducing power of glycoproteins isolated from *F. serratus*/Termamyl, *F. serratus*/Viscozyme and *F. vesiculosus*/Termamyl at 5 mg/ml is comparable to that of ascorbic acid, which is a one of the strongest reducing agents used as antioxidant. Taken together, results obtained with the above mentioned antioxidant assays suggest that the isolated glycoproteins possess a combination of different anti-oxidative properties, which may make them able to prevent lipid oxidation by different modes of action, which will be an advantage when applied in complex food and biological systems. The potential anti-oxidative activity was also investigated in a liposomal model system mimicking the cell membrane in a biological system. The % inhibition of lipid oxidation in this model system was found to be promising as all the glycoproteins except the *F. vesiculosus* prepared by the modified method showed similar or better inhibition than BHT at 0.2mg /ml concentration. This finding supports the results obtained with the antioxidant assays.

Glycoproteins isolated from macro-algae have previously been reported to be implicated in the control of apoptosis and cell proliferation, as antioxidant, for DNA and liver protection against cancer. Moreover, bovine lactoferrin which is a glycoprotein, which also has high metal chelating activity, has been shown to have antimicrobial and antioxidant and intestine associated immune functions. The glycoproteins isolated by the present method may be expected to have similar biological properties. As mentioned above the results obtained in the liposomal model system support the assumption, that the glycoprotein may have anti-oxidative effects in biological systems.

The isolated glycoproteins may find applications as dietary supplements due to their potential biological activities, particularly anti-oxidative activities. They may also be applied in foods products such as fish oils, mayonnaise, dressing and other food products rich in polyunsaturated fatty acids. The glycoproteins may be particularly efficient in food products where metal catalyzed oxidation is pronounced, e.g. mayonnaise. A third application area may be skin care products, where the isolated glycoproteins may play a dual role by providing protection against oxidation in the skin care product as well as in human skin upon exposure to sunlight.

Examples

Example 1

Samples and pre-treatment:

The brown seaweeds *Fucus vesiculosus* and *Fucus serratus* were collected from the sandy beaches of Hou havn (55°54'39N 10°14'59E) in Denmark. Immediately hereafter, the rinsed seaweeds were placed in a freezer (-40 °C) until further use. The seaweed samples were freeze-dried for 2 days and ground to a fine powder and passed through a 0.5 mm sieve to get a uniform powder. The powdered seaweeds were stored at -80 °C under vacuum packing.

The enzymes used for the present examples were kindly donated by Novozymes (Novozymes A/S - Bagsværd, Denmark). L-α-phosphatidyl choline, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), thiobarbituric acid (TBA), ascorbic acid, ethylene diamine tetra acetic acid (EDTA) were obtained from Sigma-Aldrich (Steinheim, Germany). All other reagents were of analytical grade obtained from Merck (Darmstadt, Germany).

Enzymatic digestion:

F. vesiculosus and *F. serratus* powders were digested either by Viscozyme (a multi-enzyme complex containing arabanase, cellulase, β-glucanase, hemicellulase and xylanase), Alcalase (an endo-peptidase) or Termamyl (a heat stable alpha-amylase). For this, 2 g of freeze dried powder was suspended in 100ml buffer and adjusted to the corresponding optimum pH (for Viscozyme: 0.1M acetate buffer pH 4.5; Alcalase: 0.1M phosphate buffer pH.8; Termamyl: 0.1M phosphate buffer pH 6). The flasks were placed in a shaking water bath corresponding to its optimum temperature (for Viscozyme and Alcalase at 50°C and Termamyl at 60°C) and 100mg of the corresponding enzyme solution was added and incubated for 20h. The reaction was terminated by keeping the sample at 100°C for 10 minutes and cooled in an ice bath. The mixtures were centrifuged at 2800 rpm for 20 minutes, whereafter the supernatant was filtered, adjusted to pH 7 and freeze dried.

Glycoprotein isolation :

The flow diagram for the isolation procedure is shown in Figure 1.

The freeze dried enzymatic extracts were re-dissolved in 200ml distilled water and homogenized by using an Ultra Turrax Homogenizer in order to get a uniform solution, which was then transferred to a separating funnel. Lipids present in the aqueous solution were extracted with 100ml of n-hexane and the hexane phase was discarded. The aqueous phase was then extracted 3 times with 200 ml ethyl acetate in order to remove phenolic compounds. Then, the pH of the aqueous phase was adjusted to 5 and again extracted 3 times with 200 ml of ethyl acetate. The pH of the aqueous phase was adjusted to a value

between 1.5 and 2 by adding an appropriate amount of 6M HCl so that protein components precipitated. The remaining aqueous phase was again extracted with 200 ml ethyl acetate as described above in order to remove any residual phenolic compounds. The water phase after ethyl acetate extraction was evaporated for 15 minutes in a rotary evaporator at 40 °C to remove any leftover ethyl acetate. Then, the water phase was centrifuged at 10000rpm for 10 minutes and the precipitate was freeze dried. To about 2g of the pH precipitate fraction was added 100 ml of cold 6M urea and pH was adjusted to 8.5 by using 0.4M potassium bicarbonate. The solution was kept for overnight with stirring in a magnetic stirrer at 4 °C and centrifuged at 10,000 rpm for 10 minutes at 4 °C and the pellet was discarded. This step removes any non-proteinacious matter. The quantity of the supernatant was measured and a 100% TCA solution was added to make a final concentration of 5-10% V/V TCA which precipitates glycoprotein. This mixture was kept at 4 °C for 12 hours and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded and the precipitate was dissolved in a small quantity of water. The pH was adjusted to 7.2 to 7.5 so that the precipitate completely dissolved in water and then the solution was centrifuged at 10,000rpm for 3 minutes to remove any un-dissolved matter. The supernatant was filled in a dialysis membrane and dialyzed against water for 24 hours (molecular cut-off dialyzing tubes 1kDa). The pH of the dialyzed samples was adjusted to 1.5-2.5 using 6N HCl and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the precipitate was washed with 10 ml of absolute ethanol:diethyl ether (3:1) to remove unbound carbohydrates. The solution was centrifuged at 10,000 rpm for 3 minutes and the pellet was again washed with 10 ml of absolute ethanol:diethyl ether (3:1). This step was repeated 3 times. Then, the pellet was washed with 10 ml of absolute ethanol and finally with 10 ml of anhydrous diethyl ether. After centrifugation diethyl ether was removed and the pellet was dried under nitrogen and kept in a pre-weighed container at -80 °C.

Yield:

Yield of the glycoprotein content was determined and expressed as a % of the pH precipitated fraction (Table 1, SI 1-6). The yield of glycoprotein was found to be highest for *F. serratus*/Alcalase followed by *F. vesiculosus*/Alcalase, *F. vesiculosus*/Termamyl and *F. vesiculosus*/Viscozyme. The yield of the *F. serratus* treated with Termamyl and Viscozyme was found to be low (<10%).

SI No	Sample –enzyme treated	Yield (% of the pH precipitated fraction)
1	*F. serratus*/Termamyl	9.14%
2	*F.serratus*/Viscozyme	9.8%
3	*F.serratus*/Alcalase	59.5%
4	*F. vesiculosus*/Viscozyme	20.15%
5	*F. vesiculosus*/Alcalase	32.25%
6	*F.vesiculosus*/Termamyl	31.83%
7	*F. vesiculosus*/Alcalase (modified method)	40.5%

Table 1. The yield of glycoproteins obtained as a % of the pH precipitated fractions

Example 2

Modified method for the isolation of glycoprotein:

In order to simplify the procedure disclosed in Example 1, a modified method was developed in which some of the centrifugation and freeze drying steps were eliminated. The flow diagram for the isolation procedure is shown in Figure 2.

After the enzymatic extraction and inactivation of enzyme at 100°C, the un-dissolved substances were removed by filtration and the supernatant was directly transferred to a separating funnel without freeze drying. The aqueous solution was extracted with 100 ml of n-hexane to remove lipids and the hexane phase discarded. The aqueous phase was extracted with 200 ml ethyl acetate 3 times in order to remove phenolic compounds. Then the pH of the aqueous phase was adjusted to 5 and again extracted 3 times with 200 ml of ethyl acetate. The pH of the aqueous phase was adjusted to 1.5 to 2 by using 6M HCl so that protein components were precipitated. The remaining aqueous phase was again extracted with ethyl acetate as described above in order to remove any residual phenolic compounds. The water phase after ethyl acetate extraction was evaporated for 15 minutes in a rotary evaporator at 40°C to remove any leftover ethyl acetate. Then, it was centrifuged at 10000 rpm for 10 minutes. The pellet was re-dissolved in 100 ml of cold 6M urea and pH was adjusted to 8.5 by using 0.4M potassium bicarbonate. The solution was kept for overnight stirring in a magnetic stirrer at 4 °C, centrifuged at 10,000 rpm for 10 minutes at 4 °C and the pellet was discarded. The quantity of supernatant was measured and 100% TCA solution was added to make a final concentration of 5-10% V/V TCA. This mixture was kept at 4 °C for 12 hours and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded and the precipitate was dissolved in a small quantity of water. The pH was adjusted to 7.2 to 7.5 so that the precipitate completely dissolved in water. It

was filled in a dialysis membrane and dialyzed against water for 24 hours (molecular cut-off dialysing tubes 1kDa). The pH of the dialyzed sample was adjusted to 1.5-2.5 using 6N HCl and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the precipitate was washed 3 times with 10 ml of absolute ethanol:diethyl ether (3:1) to remove unbound carbohydrates. Then, the pellets were washed with 10 ml of absolute ethanol and finally with 10 ml of anhydrous diethyl ether. After removing diethyl ether the pellet was dried under nitrogen and kept in a pre-weighed container at -80 ^{o}C.

Yield :

Yield of the glycoprotein content was determined and expressed as a % of the pH precipitated fraction (Table 1, SI No. 7). The yield of glycoprotein by modified method of F. vesiculosus- alcalase was found to be 40.5% which was slightly higher than the usual method as described in Example 1.

Example 3

Determination of Protein and sulfate content:

Protein and sulfate content was determined by the agarose method as described by Jackson and McCandless (1978). In brief, 1.1 ml of the sample was taken and 1.2 ml of 8% TCA was added. It was mixed well and 0.6 ml of appropriate agarose reagent was added (for sulfate, 0.02% agarose containing 0.5% $BaCl_2$, for protein 0.02% agarose without $BaCl_2$). It was mixed well and allowed to stand for 35 minutes. The OD was read at 500 nm in a spectrophotometer. BSA and Na_2SO_4 was used as standard for protein and sulfate respectively. The concentration of protein and sulfate in the sample was determined from the respective standard curves.

Protein content:

The protein estimation by ordinary methods like Biuret, Lowry, Bradford and Kjeldhal was found to be difficult in these samples because of the attached carbohydrate fractions. Therefore the protein was estimated by means of the agarose method by measuring the turbidity while measuring the sulfate content. The protein content in the glycoproteins varied with the enzyme used for the hydrolysis (Figure 3). *F. serratus* digested with Termamyl produced glycoprotein with a higher content of protein than the *F. vesiculosus*. The seaweeds digested with Viscozyme and Alcalase produced glycoprotein with similar protein content. Glycoprotein isolated from *F. vesiculosus* /Alcalase by the modified procedure (Example 2) produced glycoprotein with higher protein content than the normal procedure (Example 1).

Sulfate content:

Sulfate content showed a similar trend as in the case of reducing sugar (Figure 6). The glycoprotein resulted from *F. serratus* showed higher sulfate content than the *F. vesiculosus* in different enzymatic treatments. The Termamyl hydrolysates contained high sulfate content which was followed by Viscozyme and Alcalase. The glycoproteins isolated from *F. vesiculosus*/Alcalase by the modified procedure resulted in higher sulfate content than the longer procedure.

Example 4

Determination of total soluble carbohydrate and reducing sugar.

The total carbohydrate and reducing sugar contents were analysed by the phenol–sulfuric acid method and the dinitrosalicylic acid (DNS) method respectively.

Total soluble carbohydrate content:

The total soluble carbohydrate content varied with the enzyme used for the hydrolysis in various species (Figure 4). There was no significant difference in total soluble carbohydrate in glycoproteins isolated from Termamyl hydrolysates. In the case of glycoprotein produced from Alcalase hydrolysates, *F. vesiculosus* contained higher soluble carbohydrate content than *F. serratus*. Among the glycoproteins isolated from Viscozyme hydrolysates *F. vesiculosus* showed lower total soluble carbohydrates than *F. serratus*. The glycoproteins isolated from *F. vesiculosus*/Alcalase by the modified procedure resulted in low total soluble carbohydrates when compared to the longer procedure.

Reducing sugar content:

The reducing sugar content varied both with species and also with the enzyme used for the hydrolysis (Figure 5). The glycoproteins from *F. serratus* showed higher reducing sugar than *F. vesiculosus*. Glycoprotein resulting from Termamyl showed higher reducing sugar followed by Viscozyme and Alcalase. There was not much difference in reducing sugar between the longer and modified procedures.

Example 5

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly employed technique for separation of proteins according to size. Proteins with limited glycosylation are most often stained with Coomassie brilliant blue or, if high sensitivity is needed, with a silver stain. These stains, however, are far less sensitive when used for detection of highly glycosylated proteoglycans (protein glycosaminoglycans) or

glycoproteins (protein oligosaccharides), leading to weak staining or even failure of detection. This is presumably the result of steric interference by the carbohydrates with the binding of silver ions. Proteoglycans are traditionally stained with cationic dyes, such as Alcian blue or Toluidine blue, that bind to the negatively charged glycosaminoglycan side chains, whereas more neutral glycoproteins can be detected by some variation of the Schiff base reaction involving initial oxidation of carbohydrates by periodic acid and subsequent staining with Alcian blue. Here, Alcian blue is used as the primary staining agent, binding either directly to the proteoglycans or to oxidized glycoproteins subsequently enhanced by a neutral silver staining protocol.

The protein composition of the different glycoprotein samples were analysed by SDS-PAGE using precast NuPAGE ® 10% Bis-Tris gel (Invitrogen, Carlsbad, CA) with MES running buffer (50 mM MES, 50mM Tris base, 0.1% SDS, 1mM EDTA, pH 7.3) in order to separate lower molecular weight fractions down to 3 kDa. Samples with protein concentrations of 1 mg/mL were diluted 1:1 by SDS sample buffer (100 mM Tris HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 10% glycerol, 40 mM dithiothreitol (DTT)) and boiled for 3 min at 100oC. Aliquots containing 20 µg of protein were loaded into each well in the gel. SeeBlue® plus2 (Novex, 5791 Van allen Way, Carlsbad, CA) pre-stained standards were used for comparison. Electrophoresis was carried out by using the XCell SureLock™ Mini-Cell with a constant voltage of 200V for 35 min. The glycoproteins were subjected to periodic acid oxidation and subsequent Alcian Blue and silver staining.

The SDS page electrophoresis of the obtained glycoproteins is shown in Figure 11a and 11b. Figure 11a shows the initial oxidation of carbohydrates by periodic acid and subsequent staining with Alcian blue and silver stain. The gels prove that glycoproteins were present. The presence of diffuse bands or smear showed the heterogenicity of carbohydrate substitution. The highly glycosylated proteins usually move as diffuse bands or broader smears which makes the molecular weight determination impossible. In Figure 11b staining of glycoproteins by silver stain alone represented the protein component. Here only diffuse bands or smear were also observed indicating the high degree of glycosylation. As mentioned earlier this is due to steric interference by the carbohydrates with the binding of silver ions to proteins.

Example 6

2.6. Analysis of monosaccharides

400ul of 2M TFA was added to 2mg of lyophilized sample in a screw cap vial. Each vial was tightly sealed and heated to 121°C for 2h in a drying oven. After hydrolysis the vials were

cooled under tap water. Hydrolysates were lyophilized and kept at -20°C under nitrogen until analysis. Prior to analysis by HPAEC-PAD, the hydrolysates were re dissolved in 5ml of double deionised water containing 0.1% of sodium azide to prevent microbial growth. Just before injection for HPAEC analysis each hydrolysate was filtered through a 0.22um GH polypro Acrodis ® filter (Pall life sciences, Ann Arbor, USA). The monosaccharides were estimated by comparing with a mixture of monosaccharide standards (L(+) fucose, L L(+)-rhamnose, D-(-)arabinose, D-(+)galactose, D-(+) glucose, D-(+)xylose, D-(+)mannose, L-(-)-guluronic acid and D-(+)-mannuronic acid to the acid hydrolysis conditions mentioned above.

	µg/mg dry matter								
Sample	D-(+)-Fucose	L-(+)-Rhamnose	D-(-)-Arabinose	D-(+)-Galactose	D-(+)-Glucose	D-(+)-Xylose	D-(+)-Mannose	L-Guluronic acid	D-mannuronic acid
F. serratus /Termamyl	2.7 ±1.4	0	0.2 ±0.02	0.82 ±0.4	0.93 ±0.6	0	0.7 ±0.4	3.1 ±1.0	8.3 ±2.4
F. serratus/ Viscozyme	22.7 ±2.8	0	0.7 ±0.2	3.8 ±0.1	3.4 ±0.1	0.4 ±0.0	0.5 ±0.1	5.2 ±0.6	13.5 ±1.4
F. serratus /Alcalase	5.5 ±0.7	0	0.1 ±0.0	0.9 ±0.02	0.6 ±0.02	0	1.1 ±0.1	14.4 ±1.7	16.4 ±1.8
F. vesiculosus /Termamyl	14.2 ±1.2	0	0.5 ±0.1	3.1 ±0.2	2.6 ±0.1	0	2.9 ±0.1	8.1 ±0.7	15.4 ±1.5
F. vesiculosus /Viscozyme	4.5 ±0.4	0.3 ±0.04	0.14 ±0.01	1.4 ±0.2	0.8 ±0.01	0	2.4 ±0.2	12.9 ±1.9	91.7 ±11.1
F. vesiculosus /Alcalase	2.2 ±0.4	0	0.4 ±0.1	1.04 ±0.3	1.14 ±0.4	0	0.9 ±0.1	41.8 ±0.9	105.2 ±4.9
F. vesiculosus /Alcalase (modified method)	7.0 ±1.0	0	0.1 ±0.0	1.0 ±0.7	1.4 ±0.4	0	1.4 ±0.1	15.0 ±1.9	18.5 ±1.7

Table 2. The monosaccharide composition of the obtained glycoproteins

The monosaccharide composition varied with species and the enzyme used for the hydrolysis (Table 2). The content of fucose, arabinose, glucose and galactose showed a similar trend and was highest for *F. serratus*/Viscozyme which was followed by *F. vesiculosus*/Termamyl and in most cases *F. vesiculosus*/Alcalase. There was not much difference in the content of these monosaccharides among the other glycoproteins. Rhamnose was absent in all of them except in *F. vesiculosus*/Viscozyme where it was found in trace amounts. The content of mannose was found to be high for *F. vesiculosus*/Termamyl and *F. vesiculosus*/Viscozyme which was followed by *F.*

serratus/Alcalase in both improved and normal method. There was not much difference in other treatment groups. Glucoronic acid and mannuronic acid content was highest for *F. vesiculosus*/Alcalase which was followed by *F. vesiculosus*/Viscozyme. The monosaccharide composition of *F. vesiculosus*/Alcalase by modified method was quite different when compared to the longer mothod.

Example 7

Amino acid composition:

Amino acid composition varied with species and treated enzyme (Table 3). *F. serratus*/Termamyl and *F. vesiculosus*/Viscozyme contained high Arg and Glu which was followed by Asp, Leu and Phe. *F. vesiculosus*/Alcalase contained high content of Ser, Tyr, Ala and Arg. *F. vesiculosus*/Termamyl, *F. serratus*/Viscozyme, *F. serratus*/Alcalase, *F. vesiculosus*/Alcalase modified contained high levels of Lys. These glycoproteins also contained high levels of Ser, Val, Gly, Met and Asp. The modified procedure changed the composition of amino acids.

% of total	*F. serratus* /Termamyl	*F. serratus* /Viscozyme	*F. serratus* /Alcalase	*F.vesiculosus* /Termamyl	*F.vesiculosus* /Viscozyme	*F.vesiculosus* /Alcalase	*F. vesiculosus* /Alcalase (Modified method)
LYS	5.8	18.2	34.1	26.3	7.4	7.0	34.6
ARG	18.5	0	0	0	18.4	11.0	0
ALA	3.8	0	0	0	2.3	12.3	0
C-C	1.1	0	0	0	1.2	8.5	0
LEU	7.6	0.5	1.5	2.7	7.1	2.7	1.2
MET	1.1	6.9	5.5	5.9	0.7	3.8	7.0
PHE	6.6	5.8	4.9	4.1	6.4	1.6	3.2
PRO	3.9	2.9	5.4	2.1	3.7	1.9	1.6
THR	2.3	3.6	3.3	3.4	2.9	3.02	4.7
TYR	4.9	10.4	4.8	6.8	6.3	12.4	4.7
ASP	8.1	5.1	9.8	5.6	9.1	3.1	7.9
SER	4.9	14.4	10.2	12.9	4.7	13.7	9.8
GLU	16.1	3.4	5.2	2.1	11.5	6.8	3.1
HYP	0.7	0	0	0	0.4	1.5	0
VAL	3.0	11.5	10.1	13.7	5.1	3.9	7.8
HIS	2.8	0.5	0.8	1.4	3.8	2.3	0.4
TRP	0.0	0.7	1.2	2.1	0	0	4.0
ILE	3.7	4.0	0	3.8	3.7	2.0	2.4
GLY	5.6	12.0	8.8	7.2	5.4	2.5	7.4
Sum	100	100	100	100	100	100	100

Table 3. The amino acid composition of the obtained glycoproteins (as a % of total amino acids)

Screening for anti-oxidative activity

Example 8

DPPH Radical Scavenging Activity

The scavenging effect on α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical was measured by the method of Farvin et al, (2010) with some modification in order to perform it in a microplate reader. DPPH solution (0.1 mL, 0.1 mM in 95% ethanol) was mixed with 0.1 mL of protein solution (at a concentration of 0.01, 0.1, 0.5, 1.0 and 5 mg/mL) in an Eppendorf tube. The mixture was shaken and left for 30 min at room temperature. The precipitate if any formed was removed by centrifugation at 6400 x g for 3 min (Biofuge® pico, DJB labcare Ltd, Buckinghamshire, England). The reaction mixture was transferred to microplates and the absorbance of the resulting solution was measured at 517 nm using a microplate reader (Synergy 2 multi-mode microplate reader, Biotek, Germany). For blank, 0.1 mL distilled water was used instead of the sample. BHT as mentioned above was used for comparison. Radical scavenging capacity was calculated as follows.

$$\text{DPPH radical scavenging capacity (\%)} = \frac{1 - \text{Abs of sample}}{\text{Abs of Blank}} \times 100$$

The assay results showed an increase in DPPH radical activity with increase in concentration of glycoprotein. Glycoprotein obtained from *F. serratus*/Termamyl and *F. vesiculosus*/Viscozyme showed 90% DPPH radical scavenging activity at the highest concentration tested which was higher than BHT at the same concentration (Figure 7). Moreover, the other glycoproteins showed more than 50 % DPPH radical scavenging activity when tested in the highest concentration. Importantly, the modified procedure resulted in glycoprotein with a DPPH scavenging activity, which was comparable to that of the glycoprotein obtained by the original procedure.

Example 9

Iron (Fe^{2+}) Chelating Activity

The Fe^{2+} chelating activity of the fractions was estimated by the method of Farvin et al, (2010) with some modification for analysis in a microplate reader. To 100 µL of the fractions (at a concentration of 0.01, 0.1, 0.5, 1.0 and 5 mg/mL) in Eppendorf tubes, 135 µL of deionised water and 5 µL of 2 mM ferrous chloride were added. After 3 min, 5 mM ferrozine (10 µL) was added. The mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the resulting solution was measured at 562 nm in microplate reader (Synergy 2 multi-mode microplate reader, Biotek, Germany). A blank was run in the same way using distilled water instead of sample. For comparison of metal chelating activity, the metal chelator EDTA at a concentration of 200 µg/mL was used as reference. Sample

control was made for each extract without adding ferrozine. The chelating capacity was calculated as follows.

$$\text{Iron chelating activity (\%)} = \frac{1 - \text{Abs of sample}}{\text{Abs of Blank}} \times 100$$

The Fe^{2+} chelating activity of the obtained glycoprotein was very high and showed no concentration dependency. Thus, for all the glycoproteins, the Fe^{2+} chelating activity was found to be >90% irrespective of the concentrations tested and this was also the case for the glycoprotein obtained by the modified procedure. EDTA was used for comparison and it showed a concentration dependency and increased with increase in concentration. EDTA showed >90% Fe^{2+} chelating activity at a concentration above 1mg/ml only (Figure 8).

Example 10

Reducing Power

The reducing power was measured according to the method of Farvin et al, (2010) with some modifications to perform in microplate reader. To 100 µL of peptide fractions (at a concentration of 0.01, 0.1, 0.5, 1.0 and 5 mg/mL) in Eppendorf tube, 100 µL 0.2 M phosphate buffer (pH 6.6) and 100 µL of 1 % potassium ferricyanide were added. The mixture was incubated at 50 °C for 20 min and 100 µL of 10 % TCA was added into this reaction mixture. An aliquot of 144 µL from the incubation mixture was mixed with 144 µL of distilled water and 25 µL of 0.1% ferric chloride in an Eppendorf tube. After 10 min the absorbance of solution was measured at 700 nm in microplate reader (Synergy 2 multi-mode microplate reader, Biotek, Germany). Ascorbic acid at a concentration of 200 µg/mL was used as a reference as it has good reducing property. Increased absorbance (A700) of the reaction mixture indicated increased reducing power.

Reducing power showed a concentration dependency and increased with increase in concentration (Figure 9). Ascorbic acid was used for comparison which showed high reducing power of >1.2 at a concentration of 0.1mg and higher. The reducing power of *F. serratus*/Termamyl, *F. serratus*/Viscozyme and *F. vesiculosus*/Termamyl at the highest concentration tested was comparable to that of ascorbic acid, whereas the reducing power of the glycoproteins was lower than that of ascorbic acid when evaluated in lower concentrations. Interestingly, the modified procedure resulted in glycoprotein with a higher reducing power than the glycoprotein obtained with the original procedure.

Example 11

Inhibition of Lipid Peroxidation in a Liposome Model System

Liposomes were prepared from soybean phosphatidyl choline and the liposome assay is performed according to the method described by Farvin et al (2010). Lipid oxidation was performed in a model system containing 0.1 mg of phosphatidyl choline liposomes per mL of Phosphate Buffered Saline (PBS) (3.4 mM Na_2HPO_4 - NaH_2PO_4, 0.15 M NaCl, pH 7.0) and fractions at final concentrations of 0.25, 0.5, 1.25, 2.5 and 4.5 mg protein/mL. Lipid oxidation was initiated by iron redox cycling using 50 µM $FeCl_3$ and 100 µM ascorbate. The order of addition was buffer, extracts, liposome, ferric chloride and ascorbic acid. The reactants were mixed by vortexing for 2 seconds and incubated at 37 °C in a water bath for 1 hour. The liposome assay solution with distilled water instead of sample was used as control. Lipid oxidation was measured by determining the concentration of thiobarbituric acid reactive substance (TBARS) formed. In brief, aliquots (0.25 mL) of liposome suspensions were sampled into test tubes and made up to 1 mL by adding distilled water (0.75 mL). Then, 2 mL of TBA reagent (3.75 g/L TBA; 150 g/L trichloro acetic acid; HCL 0.25 mol/L; 0.1 g/L BHT) was added. The tubes were closed and heated in a boiling water bath for 15 min, immediately cooled and centrifuged (1500 x g; 10 min). A reagent blank was prepared in the same manner as mentioned above with distilled water instead of sample. BHT at a concentration of 200 µg/mL also made in a similar manner was used for comparison. The absorbance at 532 nm of the supernatant was read against a blank. The amount of TBA-reactive substances (MDA) released/mg phospholipid (PL) was calculated using the molar extinction coefficient of MDA as 1.56 x 105. The % inhibition of TBARS formation was calculated as follows

$$\% \text{ inhibition} = \frac{Tc - Ts}{Tc} \times 100$$

Where Tc is the µmoles of MDA released by the control (Liposome alone) and Ts is the µmoles of MDA released by the samples.

The % inhibition of lipid oxidation in liposomal model system showed a concentration dependency and increased with increase in concentration (Figure 10). The % inhibition of lipid oxidation in liposomal model system also was found to be promising as all the glycoproteins except the *F. vesiculosus* prepared by the modified method showed similar or better inhibition than BHT at 0.2mg /ml concentration. When evaluated in the two highest concentrations, the ability of the different glycoproteins to inhibit lipid oxidation in liposomal model system was similar. When evaluated in the lowest concentration, the glycoprotein from *F. serratus*/Alcalase was the most efficient inhibitor of lipid oxidation whereas the

glycoproteins from the non-modified and modified *F. vesiculosus*/Alcalase processes were the poorest, with the modified process resulting in the lowest degree of inhibition.

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CLAIMS

1. A method for isolation of glycoproteins with anti-oxidative properties from brown algae, comprising the steps :

a) providing a powder of brown algae,
b) treating an aqueous solution of brown algae powder with a hydrolytic enzyme or a combination of hydrolytic enzymes, and
c) precipitating glycoproteins from the enzymatic extracts of brown algae powder at low pH.

2. A method according to claim 1, wherein pH in step c) is between 1 and 3.

3. A method according to claim 1 or claim 2, further comprising removal of lipids, phenolic compounds and free carbohydrates (mono-, di-, oligo- and polysaccharides).

4. A method according to claim 3, comprising the steps :

a) providing a powder of dried brown algae,
b) treating an aqueous solution of brown algae powder with a hydrolytic enzyme or combination of hydrolytic enzymes,
c) removing lipids, e.g. by extraction with hexane,
d) removing phenolic compounds, eg. by extraction with ethyl acetate,
e) adjusting pH of the aqueous solution to 1-3 to precipitate glycoproteins, and
d) removing free carbohydrates, e.g. by extraction with ethanol:diethyl ether, ethanol and anhydrous diethyl ether.

5. A method according to any one of claims 1 to 4, wherein the hydrolytic enzyme(s) is/are one or more peptidase(s), one or more carbohydrase(s), or a combination thereof.

6. A method according to claim 5, wherein the enzyme is an alpha-amylase, e.g. Thermamyl.

7. A method according to claim 5, wherein the enzyme is an endo-peptidase, e.g. Alcalase.

8. A method according to claim 5, wherein the enzyme is a multi-enzyme complex, e.g. Viscozyme.

9. A method according to any one of claims 1-8, wherein the brown algae is selected from *Fucus vesiculosus* and *Fucos serratus*.

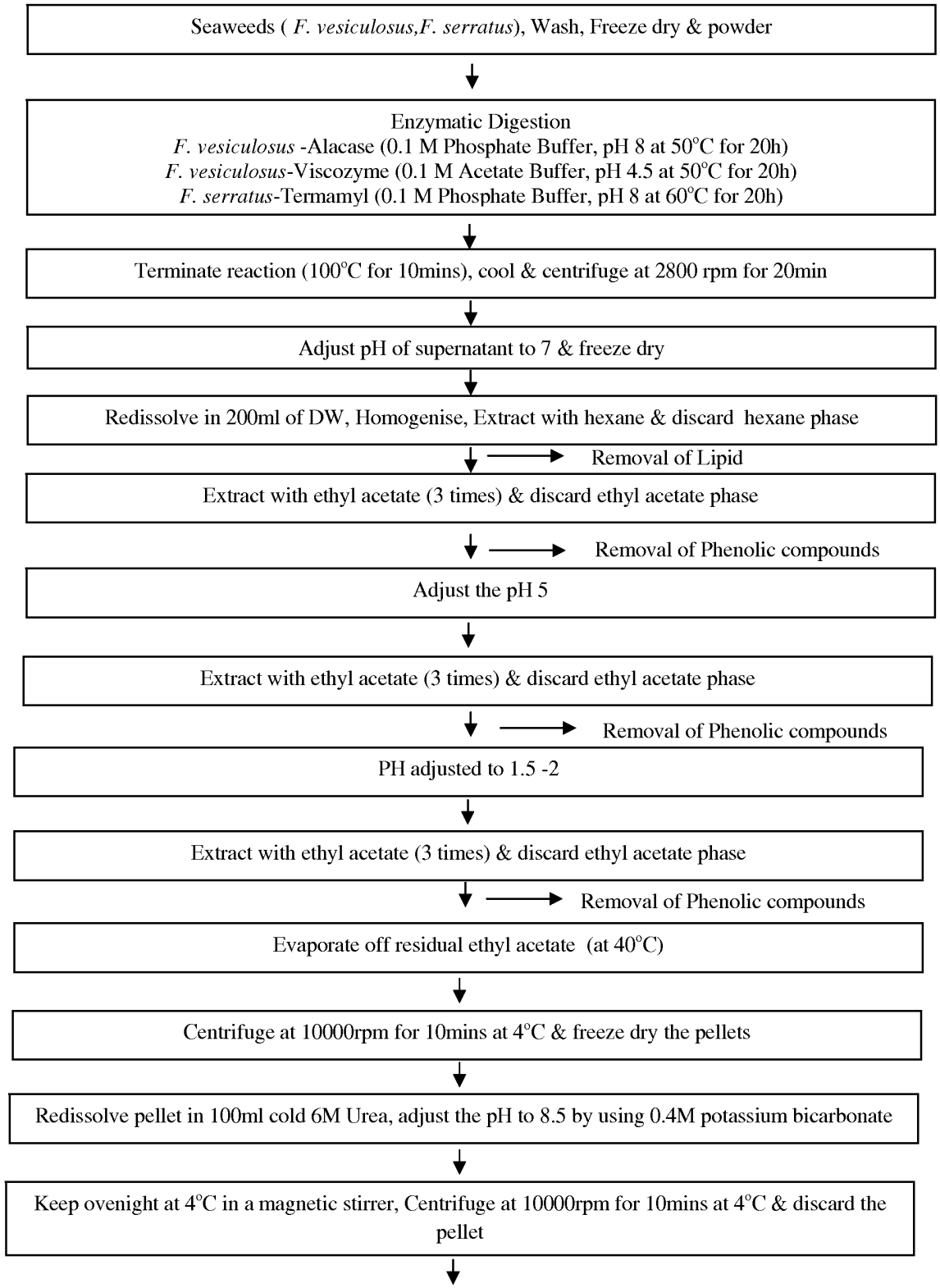
10. A method according to any one of claims 1-9, wherein the powder of brown algae is obtained by rinsing, freeze-drying and grounding brown algae collected from the sea.

11. A glycoprotein composition from brown algae obtainable in a method according to any one of claim 1 to 10.

12. A glycoprotein composition according to claim 11 or an anti-oxidative glycoprotein fraction thereof for use as an anti-oxidative agent.

13. A product comprising the glycoprotein composition or an anti-oxidative glycoprotein fraction thereof according to claim 11 or claim 12.

14. A product according to claim 13 which is a food, a pharmaceutical or a cosmetic product.


Seaweeds (F. vesiculosus,F. serratus), Wash, Freeze dry & powder
Enzymatic Digestion
F. vesiculosus -Alacase (0.1 M Phosphate Buffer, pH 8 at 50°C for 20h)
F. vesiculosus-Viscozyme (0.1 M Acetate Buffer, pH 4.5 at 50°C for 20h)
F. serratus-Termamyl (0.1 M Phosphate Buffer, pH 8 at 60°C for 20h)
Terminate reaction (100°C for 10mins), cool & centrifuge at 2800 rpm for 20min
Adjust pH of supernatant to 7 & freeze dry
Redissolve in 200ml of DW, Homogenise, Extract with hexane & discard hexane phase
Removal of Lipid
Extract with ethyl acetate (3 times) & discard ethyl acetate phase
Removal of Phenolic compounds
Adjust the pH 5
Extract with ethyl acetate (3 times) & discard ethyl acetate phase
Removal of Phenolic compounds
PH adjusted to 1.5 -2
Extract with ethyl acetate (3 times) & discard ethyl acetate phase
Removal of Phenolic compounds
Evaporate off residual ethyl acetate (at 40°C)
Centrifuge at 10000rpm for 10mins at 4°C & freeze dry the pellets
Redissolve pellet in 100ml cold 6M Urea, adjust the pH to 8.5 by using 0.4M potassium bicarbonate
Keep ovenight at 4°C in a magnetic stirrer, Centrifuge at 10000rpm for 10mins at 4°C & discard the pellet

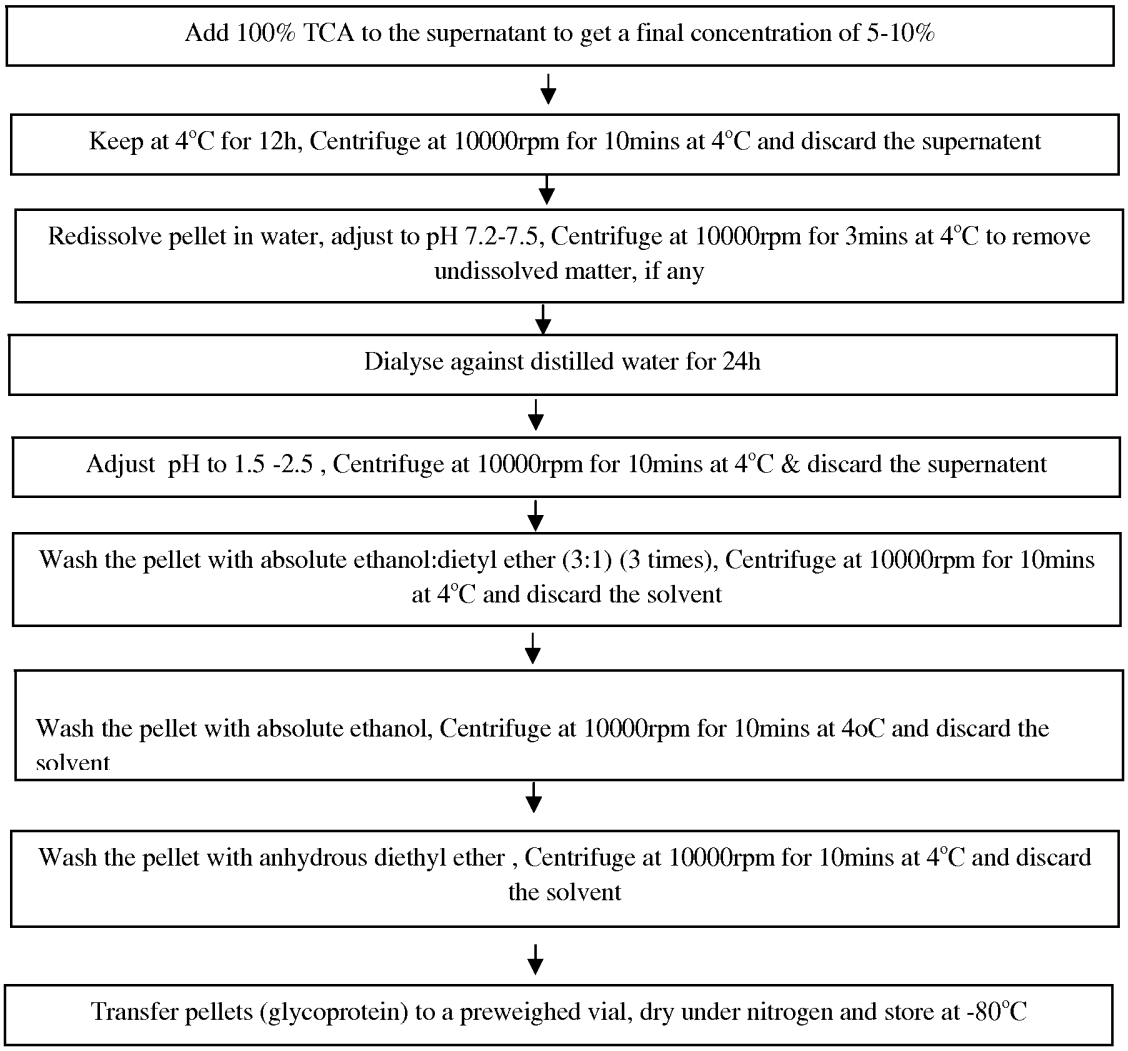
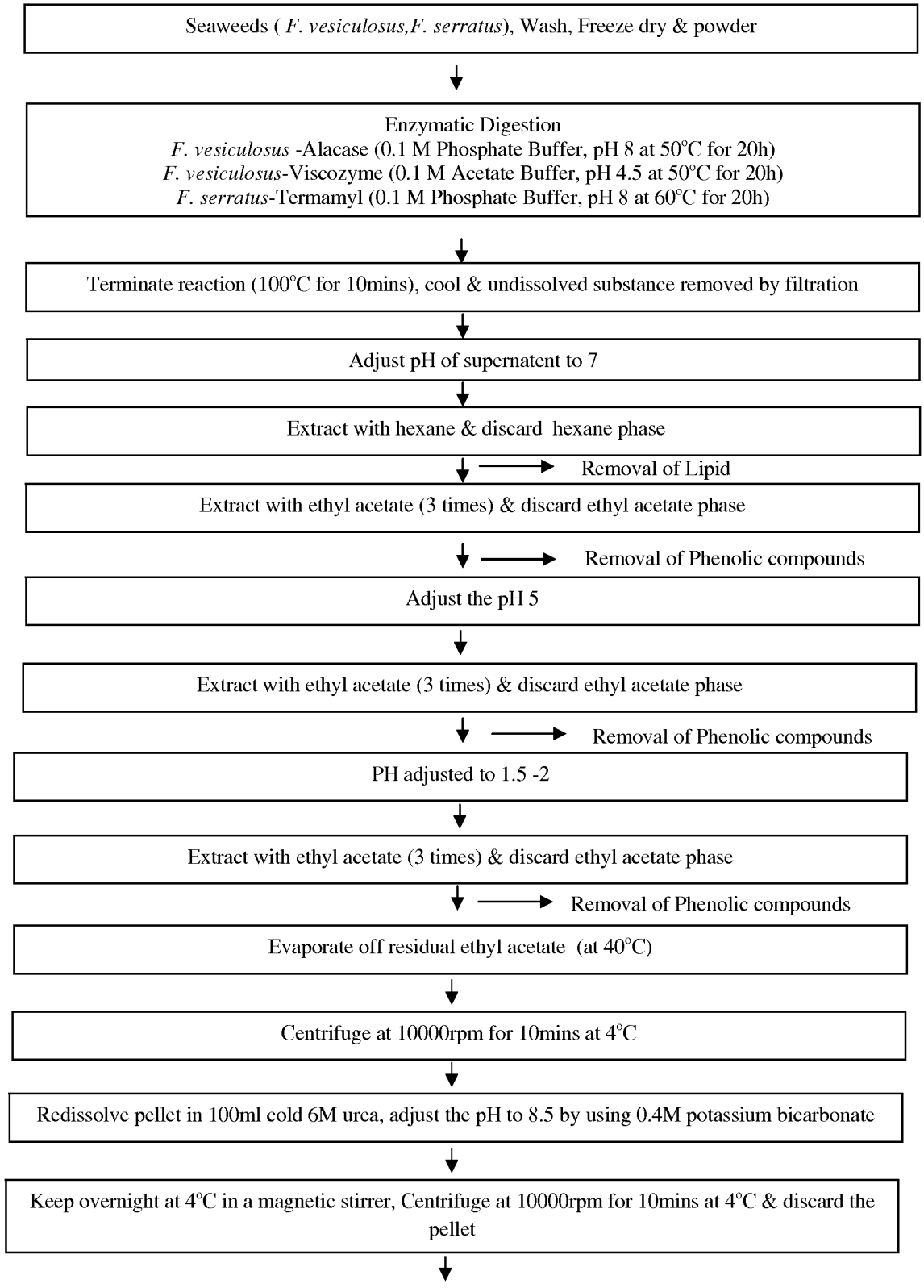

Add 100% TCA to the supernatant to get a final concentration of 5-10%
Keep at 4°C for 12h, Centrifuge at 10000rpm for 10mins at 4°C and discard the supernatent
Redissolve pellet in water, adjust to pH 7.2-7.5, Centrifuge at 10000rpm for 3mins at 4°C to remove undissolved matter, if any
Dialyse against distilled water for 24h
Adjust pH to 1.5 -2.5 , Centrifuge at 10000rpm for 10mins at 4°C & discard the supernatent
Wash the pellet with absolute ethanol:dietyl ether (3:1) (3 times), Centrifuge at 10000rpm for 10mins at 4°C and discard the solvent
Wash the pellet with absolute ethanol, Centrifuge at 10000rpm for 10mins at 4oC and discard the solvent
Wash the pellet with anhydrous diethyl ether , Centrifuge at 10000rpm for 10mins at 4°C and discard the solvent
Transfer pellets (glycoprotein) to a preweighed vial, dry under nitrogen and store at -80°C

FIGURE 1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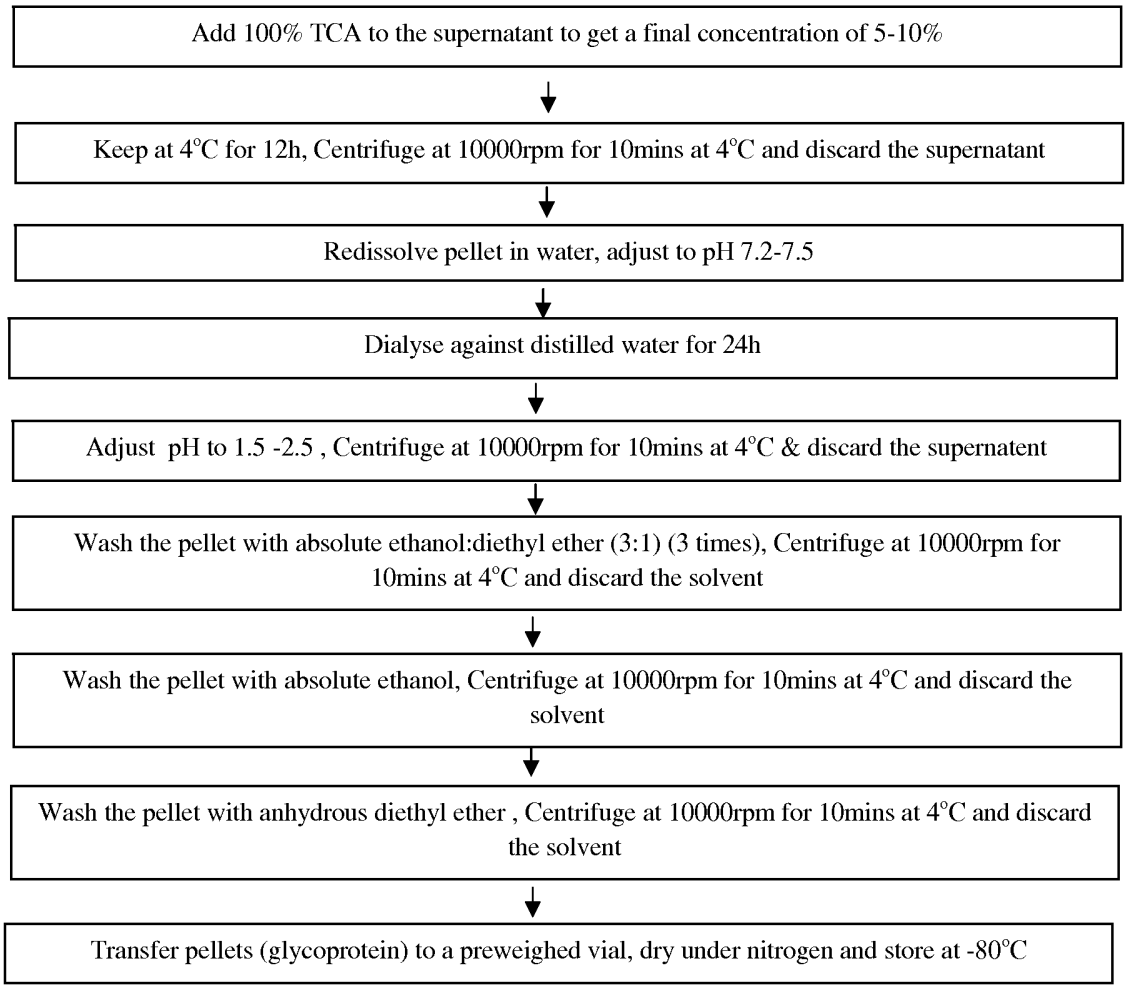
F. vesiculosus -Alacase (0.1 M Phosphate Buffer, pH 8 at 50°C for 20h)
F. vesiculosus-Viscozyme (0.1 M Acetate Buffer, pH 4.5 at 50°C for 20h)
F. serratus-Termamyl (0.1 M Phosphate Buffer, pH 8 at 60°C for 20h)
Terminate reaction (100°C for 10mins), cool & undissolved substance removed by filtration
Adjust pH of supernatent to 7
Extract with hexane & discard hexane phase
Removal of Lipid
Extract with ethyl acetate (3 times) & discard ethyl acetate phase
Removal of Phenolic compounds
Adjust the pH 5
Extract with ethyl acetate (3 times) & discard ethyl acetate phase
Removal of Phenolic compounds
PH adjusted to 1.5 -2
Extract with ethyl acetate (3 times) & discard ethyl acetate phase
Removal of Phenolic compounds
Evaporate off residual ethyl acetate (at 40°C)
Centrifuge at 10000rpm for 10mins at 4°C
Redissolve pellet in 100ml cold 6M urea, adjust the pH to 8.5 by using 0.4M potassium bicarbonate
Keep overnight at 4°C in a magnetic stirrer, Centrifuge at 10000rpm for 10mins at 4°C & discard the pellet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Redissolve pellet in water, adjust to pH 7.2-7.5
Dialyse against distilled water for 24h
Adjust pH to 1.5 -2.5 , Centrifuge at 10000rpm for 10mins at 4°C & discard the supernatent
Wash the pellet with absolute ethanol:diethyl ether (3:1) (3 times), Centrifuge at 10000rpm for 10mins at 4°C and discard the solvent
Wash the pellet with absolute ethanol, Centrifuge at 10000rpm for 10mins at 4°C and discard the solvent
Wash the pellet with anhydrous diethyl ether , Centrifuge at 10000rpm for 10mins at 4°C and discard the solvent
Transfer pellets (glycoprotein) to a preweighed vial, dry under nitrogen and store at -80°C

FIGURE 2

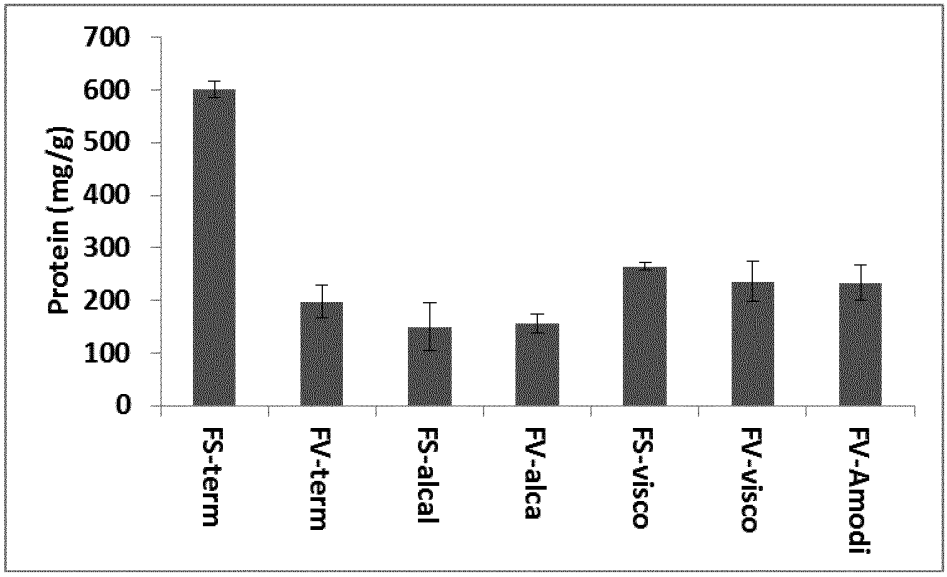

FIGURE 3

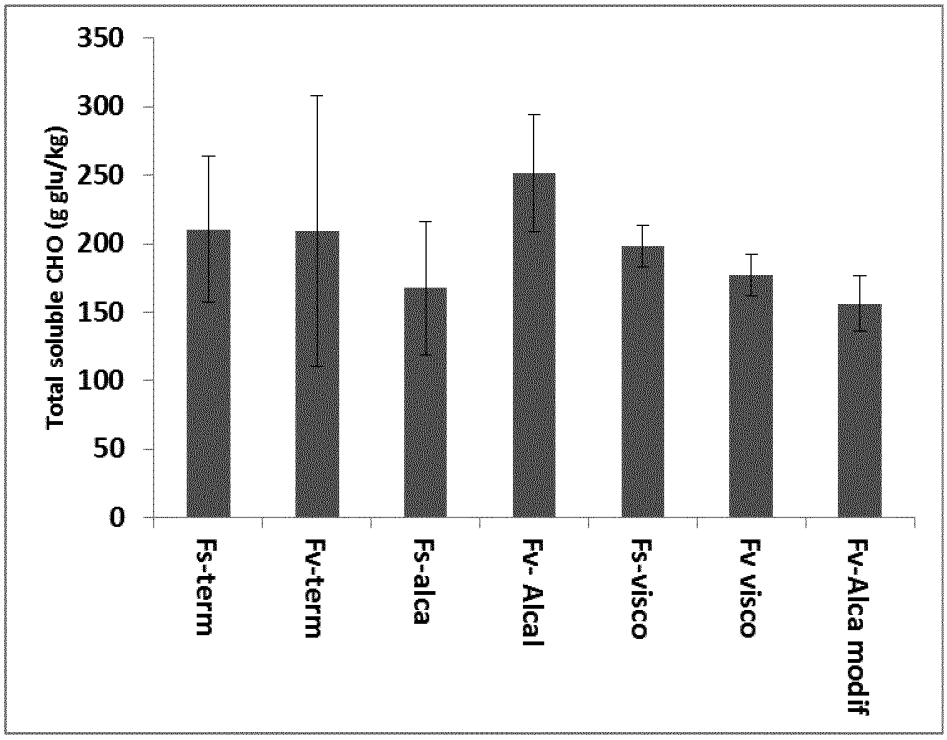

FIGURE 4

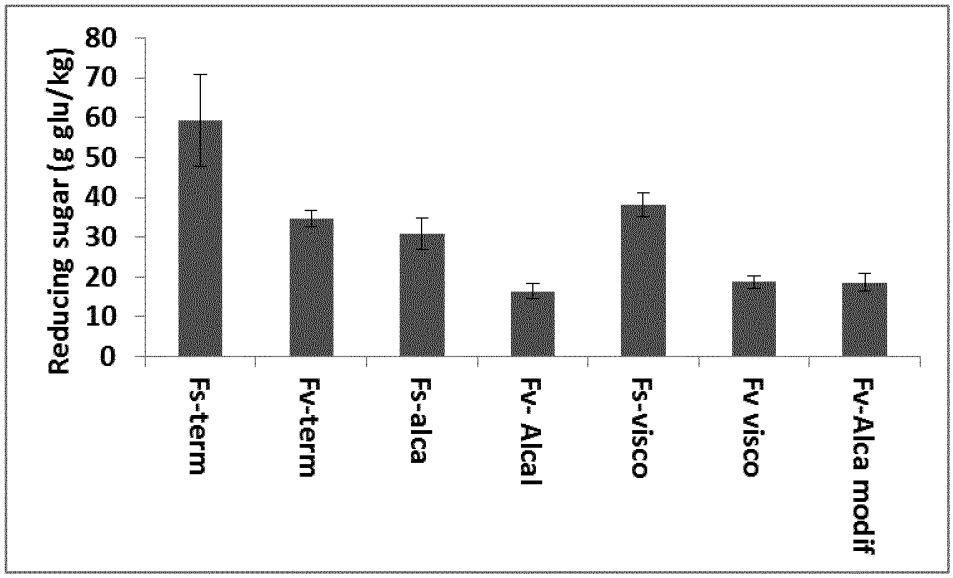

FIGURE 5

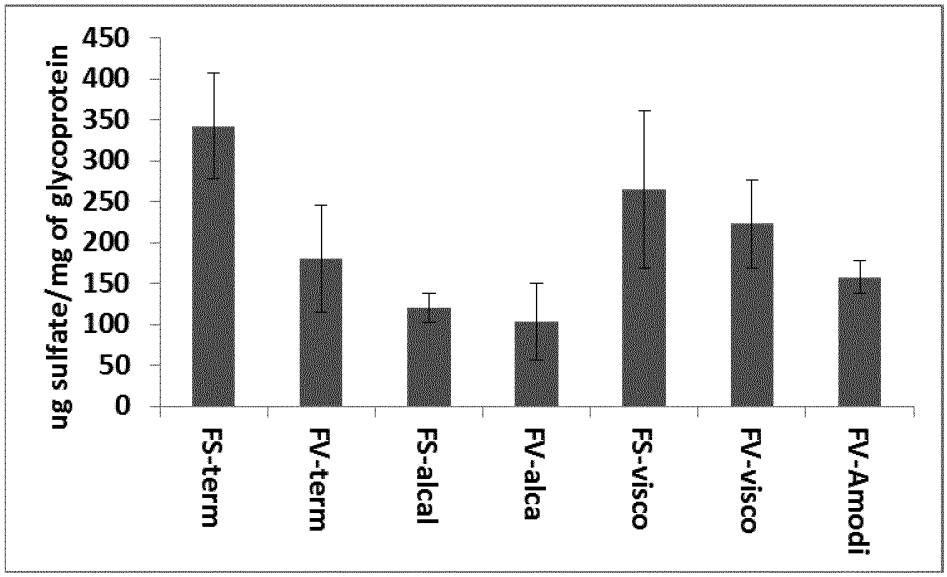

FIGURE 6

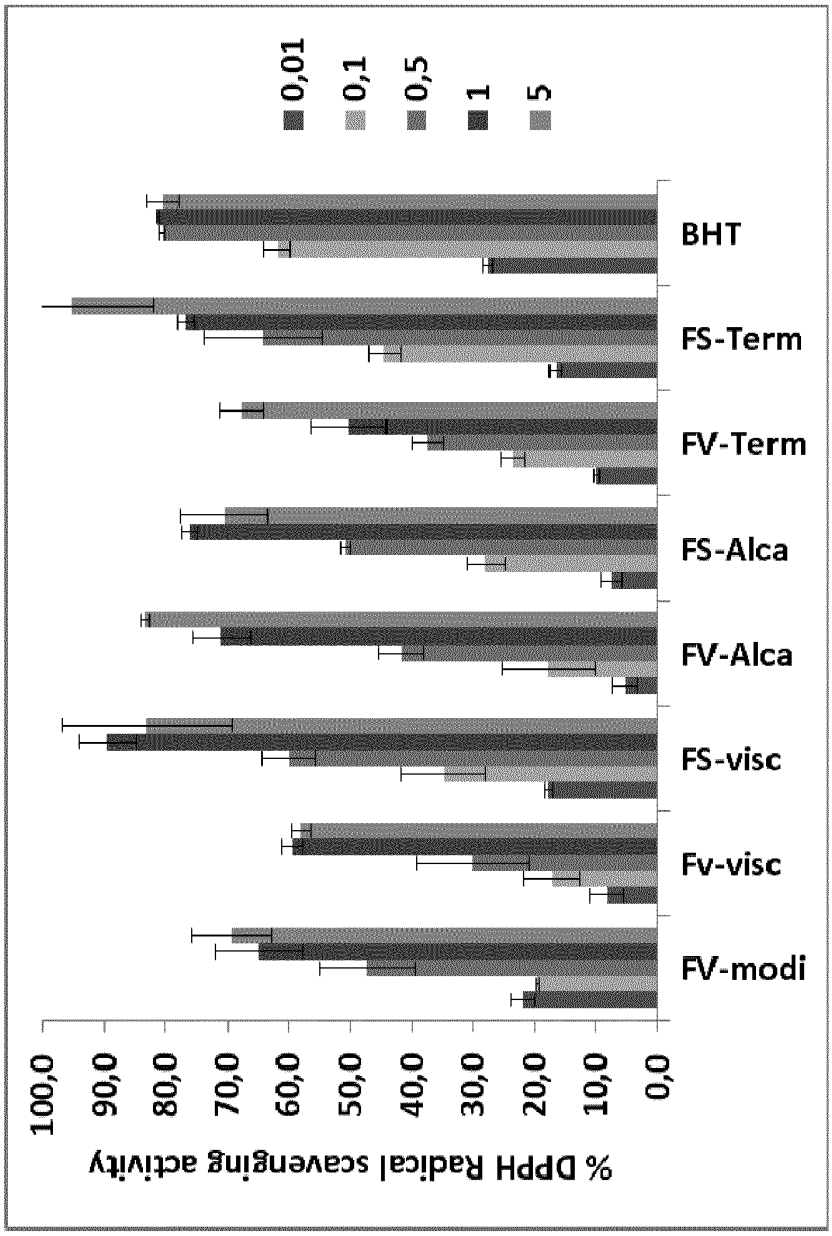
0,01
0,1
0,5
1
5
BHT
FS-Term
FV-Term
FS-Alca
FV-Alca
FS-visc
Fv-visc
FV-modi
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% DPPH Radical scavenging activity

FIGURE 7

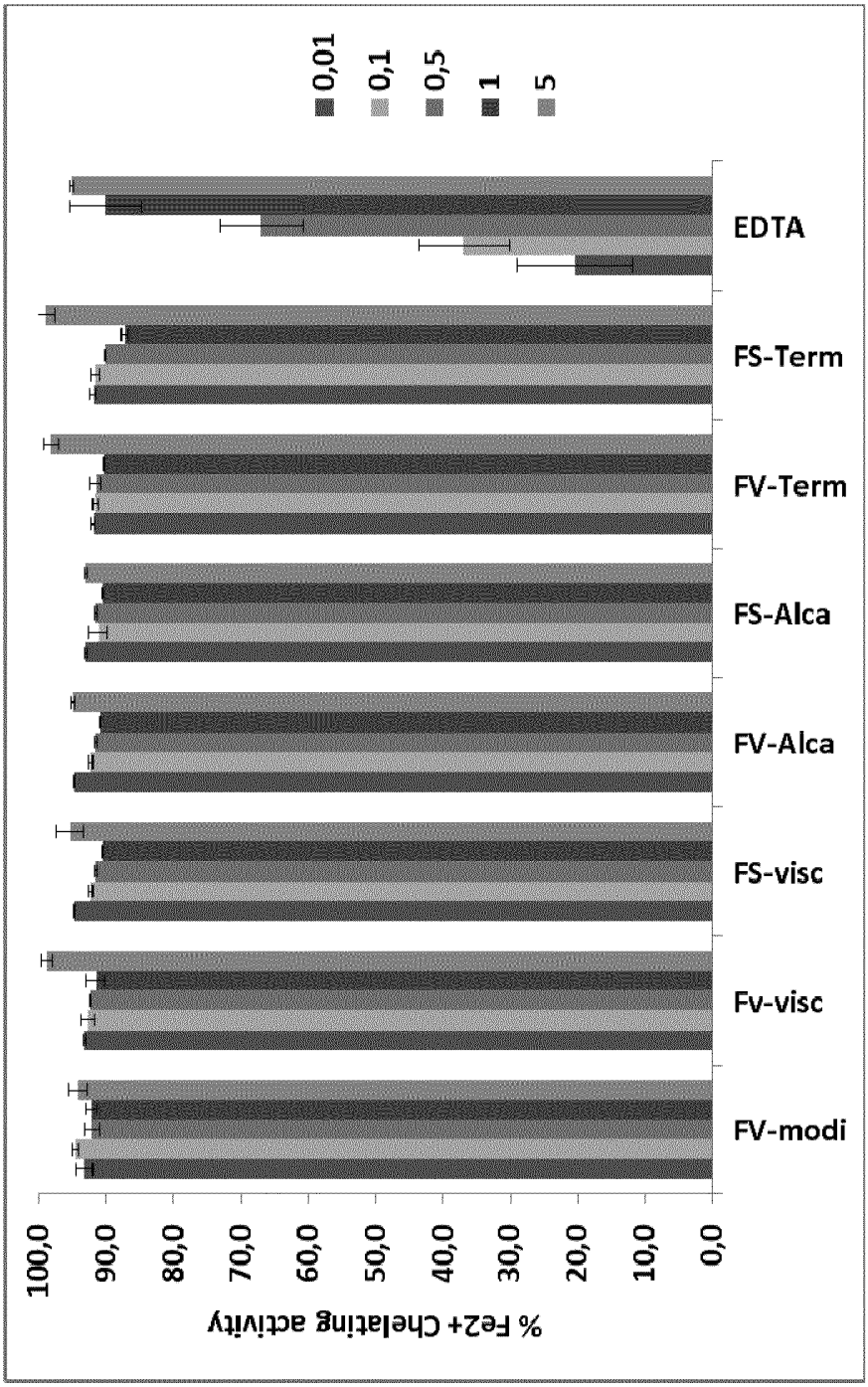
0,01
0,1
0,5
1
5
EDTA
FS-Term
FV-Term
FS-Alca
FV-Alca
FS-visc
Fv-visc
FV-modi
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Fe2+ Chelating activity

FIGURE 8

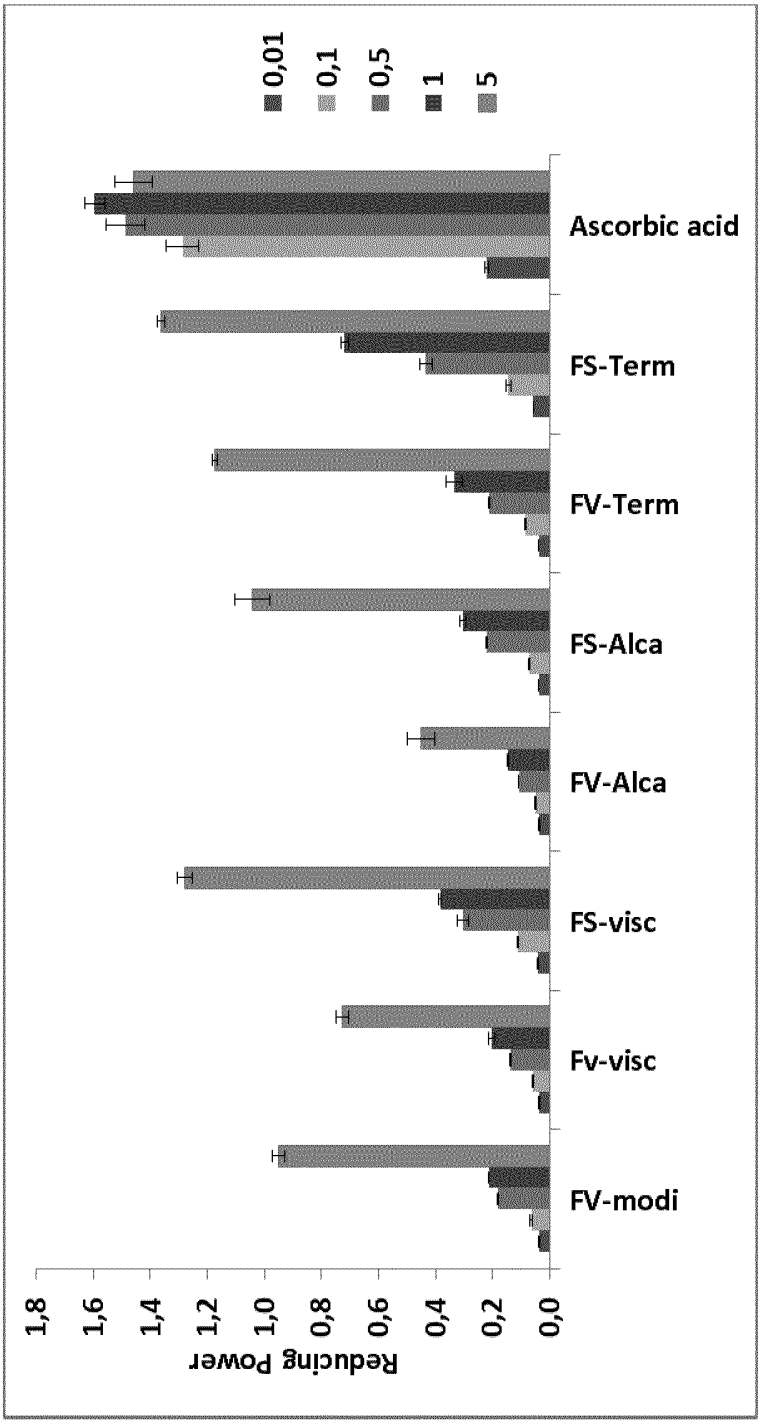
0,01
0,1
0,5
1
5
Ascorbic acid
FS-Term
FV-Term
FS-Alca
FV-Alca
FS-visc
Fv-visc
FV-modi
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Reducing Power

FIGURE 9

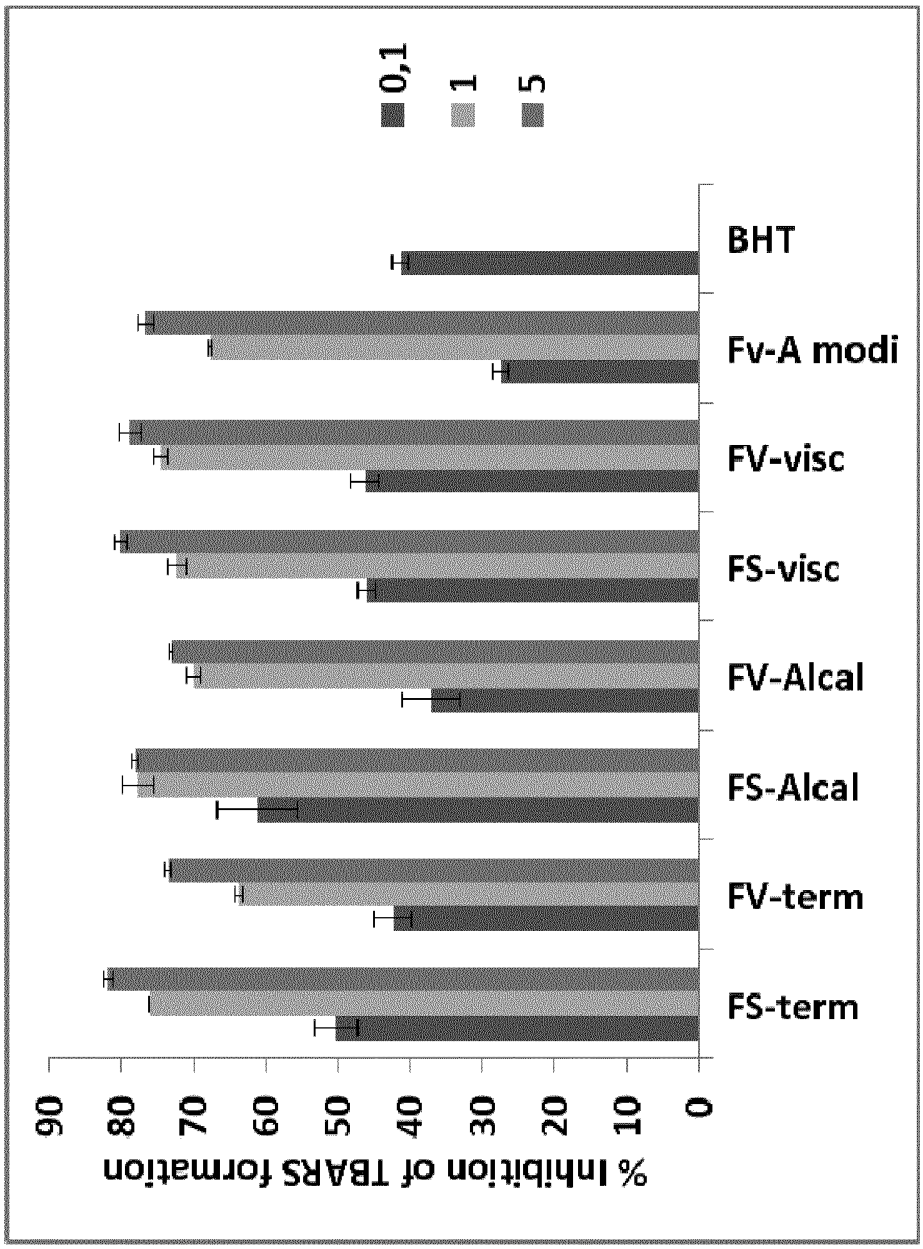
0,1
1
5
BHT
Fv-A modi
FV-visc
FS-visc
FV-Alcal
FS-Alcal
FV-term
FS-term
90
80
70
60
50
40
30
20
10
0
% Inhibition of TBARS formation

FIGURE 10

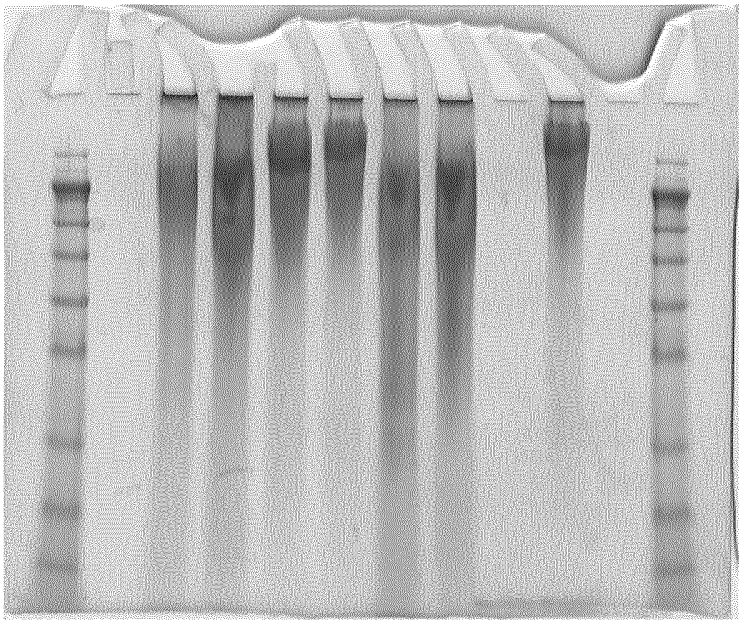

FIGURE 11a

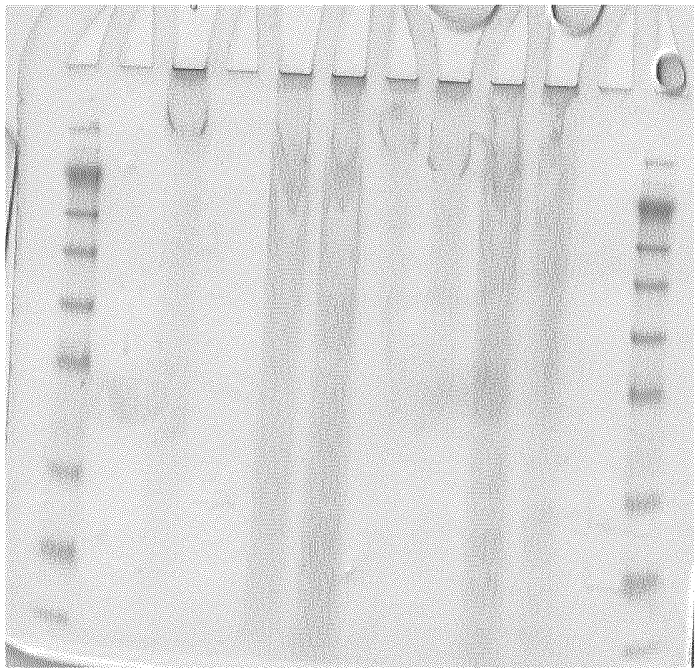

FIGURE 11b

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/064855

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P21/00 C07K1/36 A23L1/305 A61K38/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C07K A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUN-YOUNG KIM ET AL: "Antioxidant and DNA protection activities of a glycoprotein isolated from a seaweed, Saccharina japonica", INTERNATIONAL JOURNAL OF FOOD SCIENCE AND TECHNOLOGY, vol. 47, no. 5, 20 May 2012 (2012-05-20), pages 1020-1027, XP055089187, ISSN: 0950-5423, DOI: 10.1111/j.1365-2621.2012.02936.x cited in the application	11-14
Y	abstract	1-14
	Isolation of glycoprotein from S. japonica; page 1021, left-hand column	

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[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 October 2014	20/10/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van de Kamp, Mart

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Takeo Yoshiie ET AL: "Structural features of N-glycans of seaweed glycoproteins: predominant occurrence of high-mannose type N-glycans in marine plants", Bioscience, biotechnology, and biochemistry, 7 October 2012 (2012-10-07), pages 1996-1998, XP055089344, Japan DOI: 10.1271/bbb.120463] Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pubmed/23047116 [retrieved on 2013-11-20] cited in the application	11-13
Y	abstract	1-13

X	HWANG H J ET AL: "Effect of a glycoprotein from Hizikia fusiformis on acetaminophen-induced liver injury", FOOD AND CHEMICAL TOXICOLOGY, PERGAMON, GB, vol. 46, no. 11, 1 November 2008 (2008-11-01), pages 3475-3481, XP025587815, ISSN: 0278-6915, DOI: 10.1016/J.FCT.2008.08.032 [retrieved on 2008-09-02] cited in the application	11,13,14
Y	abstract	1-11,13, 14

X	RICE E L ET AL: "AN IMPROVED METHOD FOR THE EXTRACTION AND ELECTROPHORESIS OF PROTEINS AND ACTIVE ENZYMES FROM FUCALEAN MACROALGAE PHAEOPHYTA", PHYCOLOGIA, vol. 26, no. 2, 1987, pages 235-246, XP008165930, ISSN: 0031-8884 cited in the application	11,13
Y	abstract	1-11,13

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WIJESINGHE W A J P ET AL: "Enzyme-assistant extraction (EAE) of bioactive components: A useful approach for recovery of industrially important metabolites from seaweeds: A review", FITOTERAPIA, vol. 83, no. 1, 28 October 2011 (2011-10-28), pages 6-12, XP028391875, ISSN: 0367-326X, DOI: 10.1016/J.FITOTE.2011.10.016 cited in the application the whole document abstract 4. Selection of appropriate enzymes and optimum extraction conditions 5.4. Bioactive peptides from seaweeds 6. Biological activities of enzyme-assisted extracts from seaweeds	1-14
Y	HEO S J ET AL: "Antioxidant activities of enzymatic extracts from brown seaweeds", BIORESOURCE TECHNOLOGY, ELSEVIER BV, GB, vol. 96, no. 14, 1 September 2005 (2005-09-01), pages 1613-1623, XP027800770, ISSN: 0960-8524 [retrieved on 2005-09-01] abstract 5. Conclusions	1-14
Y	K H S FARVIN ET AL: "Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast", FOOD CHEMISTRY, vol. 138, no. 2-3, 15 November 2012 (2012-11-15), pages 1670-1681, XP055089332, ISSN: 0308-8146, DOI: 10.1016/j.foodchem.2012.10.078 the whole document abstract 4. Conclusions	1-14
A	WIJESINGHE W A J P ET AL: "Biological activities and potential cosmeceutical applications of bioactive components from brown seaweeds: a review", PHYTOCHEMISTRY REVIEWS, KLUWER ACADEMIC PUBLISHERS, DO, vol. 10, no. 3, 17 June 2011 (2011-06-17), pages 431-443, XP019933554, ISSN: 1572-980X, DOI: 10.1007/S11101-011-9214-4 the whole document	1,11-14

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHILPI GUPTA ET AL: "Recent developments in the application of seaweeds or seaweed extracts as a means for enhancing the safety and quality attributes of foods", INNOVATIVE FOOD SCIENCE AND EMERGING TECHNOLOGIES, ELSEVIER, AMSTERDAM, NL, vol. 12, no. 4, 5 July 2011 (2011-07-05), pages 600-609, XP028314119, ISSN: 1466-8564, DOI: 10.1016/J.IFSET.2011.07.004 [retrieved on 2011-07-12] the whole document --- SHILPI GUPTA ET AL: "Bioactive potential and possible health effects of edible brown seaweeds", TRENDS IN FOOD SCIENCE AND TECHNOLOGY, vol. 22, no. 6, 2011, pages 315-326, XP028385948, ISSN: 0924-2244, DOI: 10.1016/J.TIFS.2011.03.011 [retrieved on 2011-04-09] the whole document --- EUN-YOUNG KIM ET AL: "An improved method of protein isolation and proteome analysis with(Laminariales) incubated under different pH conditions", JOURNAL OF APPLIED PHYCOLOGY, KLUWER ACADEMIC PUBLISHERS, DO, vol. 23, no. 1, 2 July 2010 (2010-07-02), pages 123-130, XP019881955, ISSN: 1573-5176, DOI: 10.1007/S10811-010-9550-6 abstract ---	1,11-14 1,11-14 1